Bio-prospection and Bio-evaluation of *Lagotis cashmeriana* in Breast cancer

PhD Synopsis

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1. Background

Cancer ranks as a leading cause of death in every country of the world (Bray *et al*., 2021).In the year 2022, 1,918,030 new cancer cases and 609,360 cancer deaths are projected to occur in the United States. Among all the cancers, breast cancer remains the most frequently diagnosed malignancy and leading cause of cancer-associated deaths among females worldwide (Cardoso *et al*., 2020., Zaheed *et al*., 2020). Breast cancer is a heterogeneous disease which exhibit various molecular profiles with distinct clinical and biological characteristics (Perou *et al*., 2000). Based on molecular features, it is classified into 4 common groups like luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)- positive, and basal-like breast cancer (Fragomeni *et al*., 2018). Luminal A and B are hormone receptor positive (Estrogen and progesterone) and form the majority (90-95%) of hormone receptor positive, HER2 negative tumors. while compared to laminal A, luminal B cancer has higher expression of the cell proliferation marker ki67 and is associated with poor prognosis. Estrogens antagonists like tamoxifen and aromatase inhibitors are the most common drugs which are used for these two subgroups of breast cancer. For HER2-positive breast cancer, HER2 is over expressed while as estrogen and progesterone receptors are negative. Targeting HER2 by specific antibodies such as Trastuzumab is the typical treatment for this type of breast cancer. Basal-like breast cancer or triple-negative breast cancer (TNBC) is hormone and HER2-negative and is considered as the most aggressive breast cancer.

As of 2018, the death rate has decreased for female breast cancer by 41% since 1989. Despite significant declines in death rate, the incidence of breast cancer has risen more than 30% in the last 25 years (Al-Ajmi *et al*., 2018; Sanchez-Jimenez *et al*., 2019). According to Global

Cancer Statistics 2020, female breast cancer has now surpassed lung cancer as the leading cause of global cancer incidence in 2020, with an estimated 2.3 million new cases, representing 11.7% of all cancer cases and it is the fifth leading cause of cancer mortality worldwide, with 685,000 deaths.The main risk factors for breast cancer are Sex, genetic factors, use of hormone therapy, lifestyle dietary habits, and age (Garcia-Aranda *et al*., 2019). In Indian population, breast cancer (BC) has emerged as the second most common type of malignancy within the past 25 years and has one of the highest rates of the most aggressive subtype i.e; Triple Negative Breast Cancer (TNBC) (Torre L.A., 2016). The rate of TNBC in India is almost double than that of the United States (US) with estimates as high as 28-33% of all breast cancer in India compared to 12–15% in the US (Thakur *et al*., 2018). Characterized by the lack of estrogen, progesterone, and HER2 Neu receptors, TNBC lacks treatment options for targeted therapy and has a higher fatality rate as compared to other Breast Cancer subtypes which tends to occur in younger patients (women under age 50) and women of African descent (Foulkes *et al*., 2010). Breast cancer management strategies differ depending on its mass, site, whether it has extended to other organs of the body and the physical condition of the individual. Currently the treatment options for TNBC are limited. Despite great advancements in therapeutic strategies, around 30% of the patients with primary breast cancer will ultimately progress to the metastatic stage of this disease (Redig *et al*., 2013), and the 5-year survival for metastatic breast cancer is below 30% (Bonotto *et al*., 2014). Therefore, it is urgent to explore alternative therapeutics to achieve better responses in different breast cancer subtypes. Present management strategies for breast cancer include targeted therapies, hormonal treatment, radiation therapy and surgery.

Various limitations in the conventional therapy including high cost, and high toxicity of present anticancer drugs has faced a serious challange to all the researchers to design and develop an alternative, ecofriendly, biocompatible, and cost effective strategy in a greener

way (Iqbal *et al*.,2017). A large number of anti-cancer drugs have been produced from natural sources or compounds derived from these natural sources (Cragg *et al*., 2006). Among these natural sources, plants have been a valuable source of approved anti-cancer drugs, which are mostly secondary metabolites (phytochemicals) or their derivatives (Seca *et al*., 2018). Phytomolecules are expected to revolutionize cancer treatment in the next decade (Iqbal *et al*., 2017). In light of this, significant efforts are currently devoted for exploration of medicinal plants for discovery of novel active compounds which are less toxic and effective anti-cancerous agent(s). Over the last few years, there has been a noticeable increase in interest in phytochemical and pharmacological studies concerning various species of the genus Lagotis.

2. Aim: To isolate and characterize the marker compounds from *Lagotis cashmeriana* and check their antiproliferative activity in breast cancer cell lines.

3. Objectives:

1. Collection and conservation of germplasm of *Lagotis cashmeriana*.

2. Preparation of extracts and their activity in breast cancer cell lines.

3. Qualitative and quantitative estimation of secondary metabolites.

4. Isolation and characterization of marker compounds from *Lagotis cashmeriana*.

5. Antiproliferative activity of marker compounds in the Breast cancer cell lines.

4. Review of literature:

Breast cancer is a malignant tumor that starts in the cells of breast. Breast is an organ that sits on the top of upper ribs and chest muscles and is mainly made up of glands, ducts and fatty tissues. Embedded in the breast fatty and fibrous tissue there are 15 to 20 glands called lobes, each of which has many small lobules that produce milk and ducts are small canals that come out from lobules and carry milk to the nipple. If malignancy develops in lobules it is called lobular carcinoma and if it originates from ducts it is called ductal carcinoma. Based on histology, breast cancer are of different types in which common ones are DCIS, LCIS (non-invasive) and IDC, ILC (invasive). Invasive ductal carcinoma is the most common and constitutes 40-75% of all invasive breast carcinomas. Invasive lobular carcinoma represents about 5-15% of all newly diagnosed cases and generally affects women of advanced age. Based on molecular classification breast cancer if of four subtypes; luminal A 30-40%, Luminal B 20-30%, human epidermal growth factor receptor 2 (HER2)- positive 12-20%, and basal-like breast cancer (TNBC) 15-20% of all invasive breast cancer (Fragomeni et al., 2018). As per WHO, every year around 2.1 million women are detected with breast cancer and it is one of the most invasive cancer in women, contributing around 15% death worldwide (Dubey *et al*., 2022). It is assumed that one in 8 women in world will develop mammary gland cancer and only 5 to 10% of all cancers of this cancer are caused by genetic disorder, while 90-95% is connected to environmental factors and lifestyle (Castello *et al*., 2015).

International status of breast cancer: Breast cancer (BC) is the most common incident sites of cancer in women worldwide (Jemal *et al*., 2011; Stewart *et al*., 2017). Asia has 44% of the world's BC deaths with 39% of overall new BC cases diagnosed (Berry *et al*., 2018) .In United States Breast cancer is the most common cancer in women except for skin cancer. Breast cancer prognosis in the U.S. population varies significantly based on molecular

subtypes and associated clinical and demographic features (Howlader *et al*.,2018). From 2010 to 2016, 72.6% women diagnosed had luminal A, 11.2% luminal B, 4.8% ERBB enriched, 11.3% had TNBC subtypes (Acheampong et al., 2020). In recent years, luminal A and luminal B breast cancer incidence has increased, whereas triple-negative breast cancer trends have gradually declined for certain age and racial/ethnic groups. From 2010 to 2016, incidence rates of luminal A breast cancer increased in non-Hispanic White and Asian/Pacific Islander women aged 40 to 69 years, and in non-Hispanic black women aged 55 to 69 years, for luminal B breast cancer in non-Hispanic White and Hispanic women of all ages, and for ERBB2-enriched breast cancer in non-Hispanic White women aged 25 to 39 years. Incidence rates for triple negative breast cancer decreased in non-Hispanic Black and non-Hispanic White women aged 40 to 69 years.

According to the American cancer society estimates, 43,250 women will die from breast cancer for year 2022, about 287,850 new cases of invasive breast cancer will be diagnosed in women and 51,400 new cases of DCIS will be diagnosed in US. It has been seen that the incidence rates have been increased by 0.5% per year since mid 2000s and mortality rate has decreased by 1%. Advances in screening and treatment paradigms for breast cancer have led to an overall decline in mortality rate. The survival rate depends on stage of breast cancer at diagnosis, among other factors. The five-year survival rate for patients diagnosed with Stage IV breast cancer is 22%, for Stage III is 72% and Stage II is greater than 90%. HR+/HER2– status is the most common molecular subtype, accounting for two-thirds of US female breast cancer cases. In addition to advancements in treatment options over time, prognosis of breast cancer is influenced by factors that indicate growth, invasion, and metastatic potential of disease, thereby informing disease course and clinical outcome. The HR+/HER2– subtype has been associated with improved survival compared with other subtypes in the metastatic setting, also indicating some prognostic relationship between survival and receptor status.

Amongst HR+/HER2– subtype, survival is influenced by other disease-related factors such as tumor grade, site of the metastasis (eg., bone, liver, lung, or brain), prior therapy, as well as patient-related factors (eg., age, race).

National status:

With 1.3 million new cases of cancer been diagnosed annually, breast cancer is the most common women's cancer in India. India's National Family Health Survey (NFHS-4) data 2015–2016 shows that only 9.8% of women between the ages of 15 and 49 had ever undergone breast examination (Negi *et al*., 2021). In India, 14% of the cancer incidence and 11% of cancer mortalities are due to breast cancer (Bray *et al*., 2018). Approximately 25% of the female cancer cases in India are breast cancer (Malvia *et al*., 2017). The rate of incidence was found to be 25.8 in 100,000 women and the mortality rate is 12.7 per 100,000 women (2017) (Malvia *et al*.,2017). The highest rate of occurrence was found to be in Delhi (41 per 100,000 women) followed by Chennai (37.9 per 100,000 women), Bangalore (34.4 per 100,000 women), and Thiruvananthapuram district (33.7 per 100,000 women) (Malvia *et al*., 2017). The mortality-to-incidence ratio, when analyzed, was found to reach 0.66 in rural registries and 0.08 in urban registries) (Malvia *et al*., 2017). Another troubling concern about the scenario of BC in India is the increased incidence of the disease in younger Indian women (between the ages of 30 and 40). Presently, almost 48% of patients with BC in India are below 50 years of age. There is an increasing trend of BC in women between the ages of 25 and 40 in the past 25 years (Programme NCR. ICMR 2013). The surveillance trends from 2000 to 2014 based on registries from 71 countries estimated the 5-year survival ratio to be 66.1% in India which is the lowest levels among the countries included in their study (Allemani *et al*., 2018). India has one of the highest rates of the most aggressive subtype of BC referred to as Triple Negative Breast Cancer (TNBC) Torre LA (2016). The rates of

TNBC in India are almost double that of the United States (US) with estimates as high as 28% to one-third of all BC in India compared to 12–15% in the US (Thakur *et al*., 2018).

There are many methods for the treatment of cancer like tumor surgery, radiotherapy, immunotherapy, chemotherapy, cancer vaccinations, photodynamic therapy, stem cell transformation or combination thereof often accompanied by severe side effects. Such side effects include limited bioavailability, toxicity, nonspecificity, fast clearance and restriction in metastasis (Patra *et al*., 2014; Mukherjee *et al*., 2016). Chemotherapeutic agents involve cytostatic and cytotoxic drugs which have shown promising results alone or in combination with other cancer therapies to treat breast. These chemotherapeutic agents involve topoisomerase inhibitors [e.g. irinotecan (side effects include: neutropenia, sensory neuropathy, and diarrhoea) and doxorubicin (side effects include cardiotoxicity), alkylating agents e.g., carboplatin, cisplatin and cycl (side effects include: nephrotoxicity, gastrointestinal toxicity, cardiovascular toxicity, pulmonary and hematologictoxicity), microtubules acting agent e.g. vincristine, vinblastine, docetaxel and paclitaxel etc. (Weaver *et al*., 2014; Caruso *et al*., 2000). The above mentioned drugs are highly effective against cancers, but these drugs are also having some limitations: side effects, expensive, very complex, not ecofriendly and toxic. There are cells in our body which multiply rapidly under normal physiological conditions. Present a\nticancer drugs also target these rapidly dividing normal cells which is a big challenge thus, harmful side effects arise. Due to these side effects there is decreased blood production, GIT inflammation, hair loss, immune suppression, heart diseases and nervous disorders may arise. Another limitation is that these cancer cells resist to these drugs as they go through mutations. e.g., Drug resistant genes (ABCA4 and ABCA12) were over-expressed in human MCF-7 breast cancer cells respectively when docetaxel was applied. However, when phytochemical curcumin was applied in association with docetaxel down regulation of drug resistance genes was observed (Aung *et al*., 2017).

Thus, treating cancer cells by employing mono-target chemical agent is not an effective method. Therefore, based on extensive research findings, phytochemicals and their derived analogues possess most promising option for the better and less toxic cancer treatment (Singh *et al*., 2016)

Lagotis cashmeriana:

Kashmir has got huge flora of medicinal plants. About 2000 plant species are found in Kashmir. Natural products isolated from these medicinal plants are valuable sources of new drugs. Nearly, 80% of the world's population depend on traditional medicines and more than 60% of clinically approved anticancer drugs are derivatives of these medicinal plants (Cragg *et al*., 2016; Khan *et al*., 2014). There are, however, many uninvestigated natural resources which could be good sources of novel anticancer drugs. Lagotis is a genus containing about 30 species that have been traditionally used as medical plants by Tibetan folk, China. A species of lagotis called *L. brevituba* (Tibetan name is 'Honglian') whole plant of which is used as a traditional Tibetan medicine for the treatment of hypertension and nephritis and to stimulate appetite and aid digestion. But the chemical composition of only few species of Lagotis has been investigated till now. Flavanoids have been isolated from *L. brevituba* and phenylpropanoid glycosides and iridoid glycosides have been found in *L. stolonifera*. Ethanol extracts of *L. brevituba* have been reported to possess antiinflammatory, anticancer, and antimicrobial activity (Liu *et al*., 2010; Jin and Chen, 2006; Zhang et al., 2012). *L. brevituba* contains numerous compounds, including flavonoids, phenols, iridoides, phenylpropanoids, and volatile oils (Xi *et al*., 2010; Yuan *et al*., 2017; Li *et al*., 2014). It was reported that an n-butanol soluble fraction of an ethanolic extract of *L. brevituba* induced apoptosis in human gastric cancer cell lines, and the cytotoxic activity of the n-BuOH-soluble fraction and to isolate and identify the active cytotoxic compounds (Xiang *et al*., 2017). The antiproliferative activity of these isolates was evaluated in human gastric cancinoma, Colorectal cancer,

hepatic cancer cells and lung cancer cell lines. Two Flavanoids (Diosmetin, cynaroside) and mannitol have also been isolated from another species which is *L. integrifolia* (Chuluunbaatar *et al*., 2017). Diosmetin has been investigated to possess cytotoxic activity in human colorectal cancer cells; HCT-116 by inducing apoptosis of cancer cell lines and G2/M cell cycle arrest. Diosmetin has also been investigated to possess antiproliferative activity in breast cancer cell lines MDA-MB-231 (Wang et al, 2019). Cynaroside which is widely present in plants has a variety of pharmaceutical effects such as it has varying degrees of anticancer activity in different cancer cells like cervical, lung, oesophageal, and other cancer cell types. So many compounds have been isolated from different species of Lagotis but many other species which could possess natural antiproliferative compounds have remained untouched.

In Jammu & Kashmir single species *lagotis cashmeriana* Royle Rupr is found. It is commonly called as Kashmir Hare's Ear is a perennial herb of family Scrophulariaceae found in alpine slopes of Kashmir Himalayas. It is found at an altitude ranging from 3,000 to 4,000 m. It is usually found in shady and wet areas and among rock crevices. The species is used for medicinal purposes by local people but its phytochemical study has not been done yet. As compounds isolated from other species of genus Lagotis possess antiproliferative activity so it will be interesting to isolate marker compounds from *L. cashmeriana* and investigate their activity in the field of breast cancer.

5. Identification of Research gaps:

The treatment of choice for breast cancer is surgical intervention with chemotherapy, radiotherapy and hormonal therapy either in the neoadjuvant and/or adjuvant setting depending on stage of disease which are having many side effects like cost, multidrug resistance, low bioavailability etc. Because natural anticancer drugs produced by plants are

less toxic, cost effective, chemosensitizing, they are gaining interest in the treatment of cancers. Kashmir valley is a treasure of important medicinal plants which claim to have anticancer property. But much of the medicinally important flora has remained unexplored. *Lagotis cashmeriana* is one such example for which no phytochemical study has been reported yet. The isolation and characterization of the natural compounds as well as the mechanism of action of these natural compounds in drug discovery is of immense need.

Work flow to achieve the objectives

Lagotis cashmeriana

⇩

Collection
Identification
Submission

⇩

Preparation of crude extract

⇩

Cytotoxic activity of crude extract in breast cancer cell lines(MCF 7 & MDA MB 231)

⇩

Selection of medicinal plant for further analysis

⇩

Solvent extraction of plant material

⇩

ISOLATION OF MARKER COMPOUNDS

Column chromatography

⇩

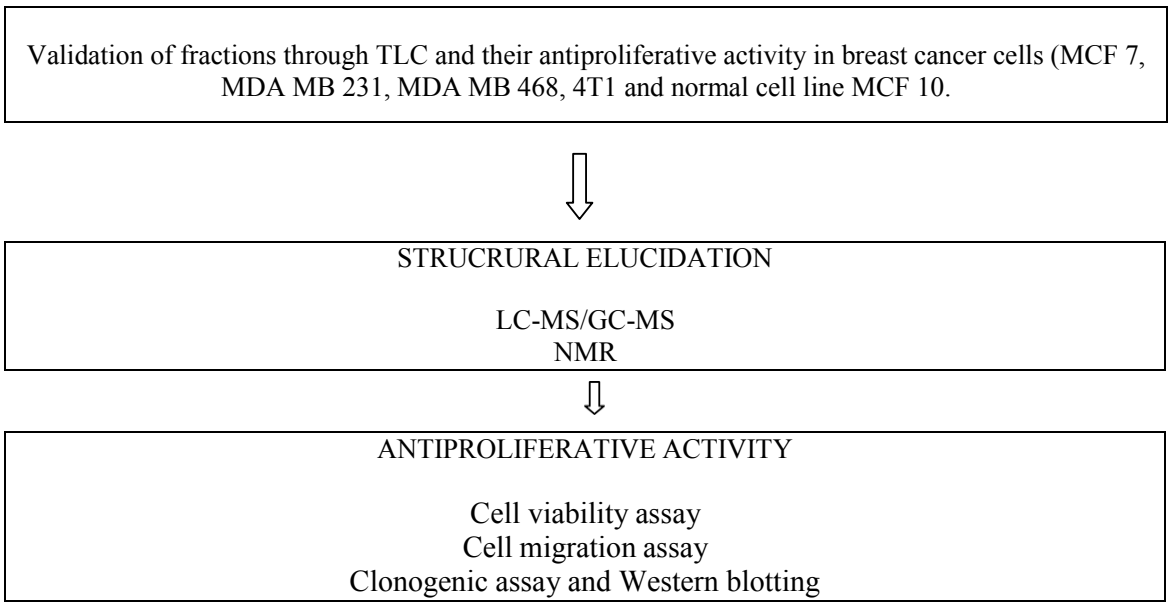
F1

F2

F3

F4, so on

⇩


Validation of fractions through TLC and their antiproliferative activity in breast cancer cells (MCF 7, MDA MB 231, MDA MB 468, 4T1 and normal cell line MCF 10.
STRUCRURAL ELUCIDATION
LC-MS/GC-MS
NMR
ANTIPROLIFERATIVE ACTIVITY
Cell viability assay
Cell migration assay
Clonogenic assay and Western blotting

6. Materials Required:

Plant material: The whole plant of *Lagotis cashmeriana* will be used.

Cell lines and maintenance of cell culture: Human breast cancer cell lines (MBA-MB-435, MDA-MB-231, 4T1 and MCF-7) and normal breast cell line (MCF-10) cells will be cultured in high glucose DMEM supplemented with 10% FBS and penicillin streptomycin at 37 degrees in 5% CO2 humified incubator. The extracts will be dissolved in 100% DMSO but the final concentration of DMSO in treated plates will be kept < 0.3% to avoid detrimental effects of DMSO.

7. Research Methodology:

Objective 1: Collection and conservation of germplasm of *Lagotis cashmeriana*

Approach: The *L. cashmeriana* will be collected from Kashmir Himalayan region and will be identified in the Centre for Biodiversity and Taxonomy, University of Kashmir, Srinagar. After proper identification, a herbarium sheet prepared at the time of collection will be submitted to KASH Herbarium, Srinagar for the issuance of voucher number.

Collection of plant: The *Lagotis cashmeriana* will be collected from Kashmir Himalayas.

Identification and Conservation of germplasm: Plants collected from Himalayan region will be identified by expert taxonomist and sheets prepared at the time of collection will be preserved in KASH Herbarium Srinagar.

Objective 2: Preparation of extracts and their activity in breast cancer cell lines.

Approach: The plant material will be will be cleaned, dried, powdered and dissolved in different suitable solvents like chloroform, ethyl acetate, petroleum ether, ethanol, methanol for extraction purpose and then antiproliferative activity of each extract will be assessed through cell viability assay. The extract possessing antiproliferative activity against Breast cancer cell lines will be selected for further study.

Plant material: Whole plant of *Lagotis cashmeriana*

Preliminary screening: Preliminary study will be done for *Lagotis cashmeriana*. A crude extract will be prepared and antiproliferative activity will be checked in breast cancer cell lines through MTT assay. The extract possessing effective antiproliferative activity will be selected for further analysis.

Preparation of plant extracts: Plant material will be cleaned, dried and powdered. Extraction will be done through hot extraction (soxhlet apparatus)

Objective 3: Qualitative and quantitative estimation of secondary metabolites.

Approach: The presence or absence of secondary metabolites will be checked through qualitative testing and the percentage concentration of secondary metabolites will be checked through quantitative testing.

A. Qualitative estimation of secondary metabolites.

Detection of alkaloids: Few quantity of the each extract will be stirred with 5 ml of 1% aqueous HCl on water bath and then filtered. To the filtrate, few drops of Dragendorff's reagent will be added; occurrence of orange-red precipitate will be an indication for the presence of alkaloids.

Detection of saponins: One gram of each portion will be boiled with 5 ml of distilled water, filtered. To the filtrate, about 3 ml of distilled water will be further added and shaken vigorously for about 5 minutes. Frothing which persisted on warming will be taken as an evidence for the presence of Saponins.

Detection of terpenoids: To 5ml of extract solution, 2 ml of chloroform will be added followed by 3ml of H_2SO_4 to form a layer. A reddish brown colouration of the inter face will be formed to show positive results for the presence of terpenoids.

Detection of phenols: To 2ml of extract solution, 2ml of ferric chloride solution will be added. The formation of deep bluish green color will indicate the presence of phenol.

Detection of cardiac glycosides (keller-killani test): Five ml of each extracts will be treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This will be under laid with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Detection of flavanoids (Shinod's test for flavanoids): About 0.5 of each portion will be dissolved in ethanol, warmed and then filtered. Three pieces of magnesium chips will then be added to the filtrate followed by few drops of concentrated HCl. A pink, orange, or red to purple colouration indicates the presence of flavonoids.

B. Quantitative estimation of secondary metabolites:

After the confirmation of presence of phenols, alkaloids, flavonoids, saponins and tannins by qualitative phytochemical tests, the plant material will be taken for quantitative testing to get the percentage concentration of secondary metabolites.

Test for alkaloids (Harborne method): 5 g of the sample will be weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol will be added, covered and allowed to stand for 4 h. This will be filtered and the extract will be concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide will be added drop wise to the extract until the precipitation will be complete. The whole solution will be allowed to settle and the precipitate will be collected and washed with dilute ammonium hydroxide and then filtered. The residue will be the alkaloid, which will be dried and weighed.

Test for saponins (Obadoni and Ochuko Method): 20 g of samples powder will be put into a conical flask and 100 ml of 20% aqueous ethanol will be added. The samples will be

heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture will be filtered and the residue will be re-extracted with another 200 ml 20% ethanol. The combined extracts will be reduced to 40 ml over water bath at about 90°C. The concentrate will be transferred into a 250 ml separating funnel and 20 ml of diethyl ether will be added and shaken vigorously. The aqueous layer will be recovered while the ether layer will be discarded. The purification process will be repeated. 60 ml of n-butanol will be added. The combined n-butanol extracts will be washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution will be heated in a waterbath. After evaporation the samples will be dried in the oven to a constant weight and the saponin content will be calculated as percentage.

Test for phenols: Spectrophotometry and Folin-Ciocalteau colorimetric method will be used. Each extract (200 µL) will be introduced into screw cap test tubes and 1.0 mL of Folin-Ciocalteau reagent (1:1 with water) and 1.0 mL of sodium carbonate (7.5%) will be added. The tubes will be vortexed and incubated for 2 h and the absorbance will be read at 726 nm using a spectrophotometer. The total phenolic content will be expressed as gallic acid equivalents (GAE) in milligrams per gram dry material.

Test for flavanoids (Aluminium Chloride method): Aluminum chloride colorimetric method will be used for flavonoids determination (Chang et al., 2002). Each plant extracts (0.5 ml of 1:10 g ml-1) in methanol will be separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It will be kept at room temperature for 30 min; the absorbance of the reaction mixture will be measured at 415 nm with a double beam UV-VIS spectrophotometer. The calibration curve will be prepared by preparing quercetin solutions at concentrations 25 to 100 µg ml-1 in methanol.

Objective 4: Isolation and characterization of marker compounds from *Lagotis cashmeriana.*

Approach: The secondary metabolites responsible for antiproliferative activity will be isolated from plant extracts and then characterized through LCMS, TLC, and NMR spectroscopy respectively.

Isolation and identification of active compound from *L. cashmeriana* extract through Column chromatography: Column chromatography is an adsorption chromatography technique which is used to separate a single chemical compound from a mixture dissolved in a fluid.Column chromatography separates substances based on differential adsorption of compounds to the adsorbent as the compounds move through the column at different rates it allow them to get separated in fractions.

The extracts in each solvent will be subjected to column chromatography over silica gel column. The column will be eluted with solvents of increasing polarity hexane, ethyl acetate, methanol, petroleum ether,and their mixtures (0, 5, 10, 20, 30, 50, 70 and 100%). A number of fractions (each 100 ml) will be collected. Similar fractions will be combined on the basis of their Thin Layer Chromatography (TLC) profiles.

Thin Layer Chromatography: To validate the presence or absence of chemical compounds in a particular fraction, Thin Layer Chromatography will be used. Similar fractions will be combined on the basis of TLC profile and sub-fractionation will be done if more than one compound will be present within a fraction.

Characterization of marker compounds: LC-MS & NMR will be used for identification and characterization of molecular structures.

LC-MS Liquid chromatography-mass spectrometry is an analytical chemistry tool used in the pharmaceutical, biotechnological, and environmental industries. This equipment combines HPLC, wherein the individual components in a mixture are first separated followed by ionization and separation of the ions on the basis of their mass/charge ratio. The separated ions are then directed to a photo or electron multiplier tube detector, which identifies and quantifies each ion. The ion source is an important component in any Mass Spectrometry analysis, as this basically aids in efficient generation of ions for analysis. To ionize intact molecules, the ion source could be APCI (Atmospheric Pressure Chemical Ionization), ESI (Electronspray Ionization). The choice of ion source also depends on the chemical nature of the analyte of interest i.e. polar or non-polar.

Nuclear magnetic resonance (NMR) is a spectroscopic technique used primarily to elucidate the structures of organic compounds, especially following synthesis or isolation of products. NMR spectroscopy is used to determine the carbon-hydrogen framework of a molecule and is based on the fact that atomic nuclei that have an angular momentum and a magnetic moment have a special property called nuclear spin.

Objective 5: Antiproliferative activity of marker compounds in the Breast cancer cell lines.

Approach: The marker compounds isolated from plant material will be selected on the basis of Lipinsk's rule of five and the activity of these marker compounds will be assessed through different assays like Cell viability assay, cell migration assay, Clonogenic assay and western blotting.

Selection of marker compounds: Compounds satisfying Lipinsk's rule of five will be selected for further analysis. Lipinsk's rule of five, also known as Pfizer's rule of five or simply the rule of five (RO5), is a rule of thumb to evaluate drug likeness. The rule describes molecular properties important for a drug's pharmacokinetics in the human body, including their absorption, distribution, metabolism, and excretion ("ADME"). However, the rule does not predict if a compound is pharmacologically active.

Antiproliferative activity of marker compounds: Antiproliferative activity will be assessed by these methods.

a) **Effect of marker compounds on breast cancer cell proliferation by MTT assay**: The antiproliferative effect of *L. cashmeriana* will be determined by the 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye uptake method. Briefly, the cells will be seeded in quintuplicate at a particular density in a 96-well plate in a final volume of 0.2 ml medium and will be incubated for 24 hrs at 37°C. Marker compounds will be added to each well and incubated for another 48 hrs. The cells will be washed with phosphate-buffered saline (PBS), and 20 µl MTT solution (5 mg/ ml) will be added to each well. After a 4hrs incubation at 37°C, 150 µl isopropanol will be added. The absorbance of MTT formazan will be measured at 490 nm. All experiments will be performed in triplicate and the percentage of cell viability will be calculated according to the following equation.

Inhibition rate (%) = OD (control) – OD (treated cells)/OD (control) × 100

b) **Effect of marker compounds on cell invasion through cell migration assay:** The effect of different marker compounds on cancer cell migration will be assessed through wound healing assay. *L. cashmeriana* extract-treated cells will be

cultured till 100% confluent monolayer and a wound will be produced using sterile pipette. Cellular debris will be removed by washing with serum free medium and will be incubated in medium supplemented with 0.1% FBS in the presence or absence of marker compound and EGF (100 ng/ ml). After 24hrs, the wound closure will be monitored and photographed at 0 and 24 h under a microscope. To quantify the migrated cells, pictures of the initial wounded monolayers will be compared with the corresponding pictures of cells at the end of the incubation.

c) **Colony formation assay**: An *in-vitro* cell survival assay based on the ability of a single cell to grow into colony. For the Clonogenic survival assay, breast cancer cells will be plated in each well of 6-well plate in 2.5 mL of culture medium. After 48 hours, cells will be treated with marker compounds at different concentrations; fresh medium with marker compounds will be replaced every 72 hours. After 15 days, colonies will be fixed in 4% formaldehyde and stained with 0.5% crystal violet and and the number of colonies will be counted manually.

d) **Western blot analysis:** Proteins extracted from cells will be immunoblotted with different antibodies. Briefly, cells treated with marker compounds or DMSO (vehicle) will be processed for immunoblotting. Lysate proteins will be resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis, transferred onto Nitrocellulose membrane and immunoblotting will be performed. After blocking with blocking buffer, the membrane will be incubated with primary antibodies overnight at 4°C. Following incubation with appropriate secondary antibody, the immunoreactive bands can be visualized using an imaging system. The blots will be re-probed with anti-actin or GAPDH antibody to correct for differences in protein loading.

8. Expected outcomes:

- Phytochemical isolation and screening of *Lagotis cashmeriana*.
- Antiproiferative activity of marker compounds from *Lagotis cashmeriana* in breast cancer cell lines.

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